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OBSERVATIONS BY MEANS OF IMMUNOFLUORESCENCE OF THE APPEARANCE AND EVOLUTION OF VACCINAL ANTIGEN IN CELL CULTURES

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The direct technique of immunofluorescence was used to observe the vaccinal virus in fibroblast cells of human embryo, in HeLa and KB cells.

The fluorescent antigen appears in the HeLa cells 6 hours after infection, and in the human embryo fibroblast cells and KB cells after 8 hours, before the histopathological modifications which appear only after 12-18 hours.

The infecting virus was detected 18 hours after the innoculation and the hemaglutinant property after 48 hours.

The appearance and evolution of the vaccinal antigen in cellular cultures was followed by means of immunofluorescence on the whole vaccinal antigen [4] [5] [6] [11] [12], as well as on its thermolabile protein fractions (antigen L), thermostable protein fractions (antigen S), the nucleoproteic fraction (antigen NP), and on the hemaglutinant lipoproteic fraction (antigen H). [9]. The work was carried out on several cellular cultures such as HeLa cells [9], KB cells, epidermal cells of larynx cancer [11], human fibroblasts [4], rabitt cornea [8], and human epidermus (vaccine and variola) [2]. The results varied according to the working conditions (cells, viral strain, strength of the innoculating material, etc.)

In the following article we will attempt a comparative study of the appearance and evolution of the vaccinal antigen in several types of cells innoculated under identical working conditions and following, in general, the histopathological aspect, the hemaglutinant property of the culture liquid and of the homogenate in the experimental cells.

Material and Method

Cellular Cultures. We have used primary cultures of human embryo as well as Hela and KB strains, cultured on Barski tubes with lamellas.

The culture media were those currently used by the Institute of Inframicrobiology /1/.

Virus. We used a dermavaccine strain /4/ adapted to human embryo fibroblast.

The cellular cultures were innoculated with virulent culture liquid which contained 100 DIC_{50}/milliliter. The innoculated and the blank tubes were examined at 4,6,8,12,16,20,24,30,46, and 72 hours after infection.

The direct technique of immunofluorescence was used. Antivaccinal antibodies were obtained from the serum of one man who was repeatedly vaccinated and revaccinated /4/.

The techniques of coupling the gammaglobulines with fluorochrom as well as the techniques used in fixing, dyeing, and examining the substances have been described in previous publications./3/.

For the study of cellular histopathological modifications the culture bearing lamellas were removed from the Barski tubes at the above mentioned intervals and dyed with hematoxylin-phloxin./1/.

In the hemaglutination reaction, binary dilutions were made from the culture media removed from tubes with infected or blank cellular cultures after previous congelation-decongelation and centrifugation. The reaction carried out in the presence of a 0.5% suspension of chicken red blood corpuscles, was read after the material was kept for 90 minutes at 37°C.

The infectious potential of the culture was studied by innoculation of human embryo fibroblast cells with the culture liquid and with the cellular homogenate resulted from infected cultures which were terminated at the intervals previously mentioned.

Results

a) Immunofluorescence. The appearance and distribution of the viral antigen in the various cells infected with the vaccine virus follows, in general, the same stages but we can note some differences according to the kind of infected cells, as follows:

In the human embryo fibroblast cells, the first appearance of virus antigen detectable by immunofluorescence occured 8 hours after infection. The antigen appeared in a few scattered cells, especially in small size cells, as very fine fluorescent granules localized in the cytoplasm in the immediate vicinity of the nucleus. Later, the number of antigen containing cells

increases. After 12 hours some cells show the presence of a large number of bigger fluorescent granules, some large and bulky or even compact fluorescent masses, localized in the cytoplasm around the nucleus or in some cytoplasmic extensions. After 16 hours, the fluorescent masses include almost the entire cytoplasm (Fig. 1). Here and there, they are bunched around or at the interior of a round or oval shaped formation, probably corresponding to the future cytoplasmic inclusions. But the virus antigen is also present outside these areas and spread through the rest of the cytoplasm. The nuclei of some cells start showing a diffuse fluorescence. At 24 hours and especially at 48 hours the cells are profoundly changed and totally and intensly fluorescent. The substance shows intensly fluorescent amorphous cellular remains.

In the HeLa cells, the appearance of the viral antigen occured 6 hours after infection in the form of a diffuse or very finely granulated fluorescence of the perinuclear cytoplasm.

At 8 hours, the granular fluorescent material starts to aggregate, especially that which is around the nucleus. In some cells the nucleus is visible, but it is difficult to determine wether it shows any viral antigen of its own. At 12 hours (Fig. 2), many cells show intense granular fluorescence of the cytoplasm, with more aggregation around the nucleus or in the form of dense fluorescent masses which seem to represent the future intracytoplasmic inclusions. Few syncytions were noticed as well as cells with 2-4 nuclei. At 24 hours the cells are profoundly modified and seem included in an amorphous fluorescent mass which starts looking like a bulky intercellular cement. (Fig 3).

In the KB cells the viral antigen appears toward the eighth hour as a fine cytoplasmic fluorescence. In the nucleus of the cells one can distinguish clearly 1-2 intensly fluorescent points which could be nucleoli. At 12 hours the immunofluorescence becomes more visible, but also in a fine granular form. At 20 hours the cells show an intense fluorescence of the cytoplasm (Fig 4). A number of nuclei show dense, well defined fluorescent masses. At 24 hours the cells are modified; some of them appear as bulky rings intensly fluorescent.

b) Histopathological Modifications. On material dyed with hematoxylin-phloxin, the cellular modifications are detectable after 16-18 hours. A marked tendency for multiplication of the cells is made obvious by the presence of numerous mitoses. Cells with 2-3 nuclei appear, as well as few syncytial formations. The shape of the nuclei is changed, they are pushed toward the periphery of the cell and the chromatin is concentrated in big lumps. In the vicinity of the nuclei we noticed the presence of oxyphile formations, well defined inclusions surrounded by a fine halo. After 24 hours the lesions are more pronounced and after 48 hours the cellular fabric shows numerous spaces in which are located round cells with picnotic nuclei.

In the HeLa and KB cells the appearance of the lesions occurs earlier.

At 12-16 hours one can notice the appearance of some larger volume cells with several nuclei; at this interval one can also notice round cells profoundly modified. At 20-24 hours the cells are crowded together in piles and at 48 hours the entire cellular mass is affected.

The appearance of the inclusions is noticed in the perinuclear zone 16-18 hours after infection and can occur either in cells with normal aspect or in the middle of the nuclei crown which forms the syncytions.

c) The hemaglutination reaction carried out with non-infected cells has given negative results, everytime.

In human embryo fibroblast cultures innoculated with vaccinal virus the reaction became positive at very low titre $\left(\frac{1}{4} - \frac{1}{8}\right)$ at 48 hours after infection.

Later, the hemaglutinant titre increases up to $\frac{1}{16}$.

In the HeLa and KB cultures innoculated with vaccinal virus, the appearance of hemaglutin was noticed at the same times as in the fibroblast cells and the hemaglutinant titre was higher $\left(\frac{1}{16} - \frac{1}{64}\right)$.

The innoculation of human embryo cellular cultures with the culture liquid and with the cellular homogenate in order to detect the newly formed infecting virus has given negative results in the first 12 hours after innoculation. The first positive results were observed in cultures terminated at 16 hours after infection.

Discussions.

The appearance of the viral antigen seems to take place a little earlier in the HeLa cells than in the human embryo fibroblast and KB cells. The antigen appears in the cytoplasm in the immediate vicinity of the nucleus, before the appearance of cytoplasmic inclusions. The antigen is not localized only in the areas corresponding to the future inclusions, but it can be present in these areas as well.

Research carried out by Carins /8/ with Thymidine showed that the cytoplasm of cells infected with vaccinal virus presents, in the immediate vicinity of the nucleus, some centers for the synthesis of desoxyribonucleic acid. These seem to correspond to the future cytoplasmic inclusions. The presence of the viral antigen detected through immunofluorescence in these very areas shows that the cytoplasm inclusions have complete virus elementary corpuscles. This reaffirms the claims of S.Nicolau /13/ who maintains that the inclusions contain elementary corpuscles.

We can assume that the viral antigen, diffusely distributed throughout the cytoplasm except for the locations of condensation of newly formed nucleic acid, does represent only incomplete virus, consisting of viral

protein. The appearance of the virus antigen in the cellular nucleus takes place later in human embryo fibroblast and Hela cells.

In the KB cells the synthesis of the viral antigen seem to take place somehow differently, namely, it appears in defined areas of the nucleus, possibly in nucleoli, and in the cytoplasm at about the same time. The appearance and development of the virus antigen in the nucleus are much more obvious in these cells than in the Hela and human embryo fibroblast cells.

Our research carried out on fibroblast type cells shows that in this type of cells the evolution of the vaccinal antigen is similar to that observed in epitholial type cells [9], [11], [12]. Indeed, we observed the fluorescent antigen first in the cytoplasm and only later in the nucleus.

The data presented show that immunofluorescence can be used to distinguish the vaccinal antigen earlier than by the use of other methods; this has been proved by us in previous articles [5], [6], [7].

While, by immunofluorescence, the viral antigen can be visualised at 6-8 hours after infection of the cells the characteristic oxyphile inclusions dyed with hematoxylin-phloxin appear only after 18-20 hours.

The hemaglutinin has been observed in the cellular homogenate after 48 hours, which confirms the work of Hirn and Schoner [10], [11], who point out the late appearance of the hemaglutinant property.

Conclusions

The use of immunofluorescence has made it possible to distinguish the intracellular viral antigen earlier than possible with other methods of investigation.

The antigen appears in the cytoplasm of Hela cells at 6 hours after infection and in KB and human fibroblast cells after 8 hours. In the cellular nucleus the antigen appears at 8 hours in Hela and human embryo fibroblast cells and at 16-18 hours in KB cells.

The histopathological modifications appear after 12-16 hours in Hela and KB cells and after 16-18 hours in human embryo fibroblast cells. Cellular inclusions appear at 16-18 hours after infection.

The infecting virus has been distinguished in the cellular homogenate at 18 hours after infection.

The hemaglutinant property was detected only at 48 hours after infection.

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Fig. 1. Human embryo fibroblast innoculated with vaccinal virus, 16 hours after infection. Ob. 40. oc. 7.

Fig. 2. HeLa cells, 12 hours after innoculation with vaccinal virus. Ob. 40, oc.7.

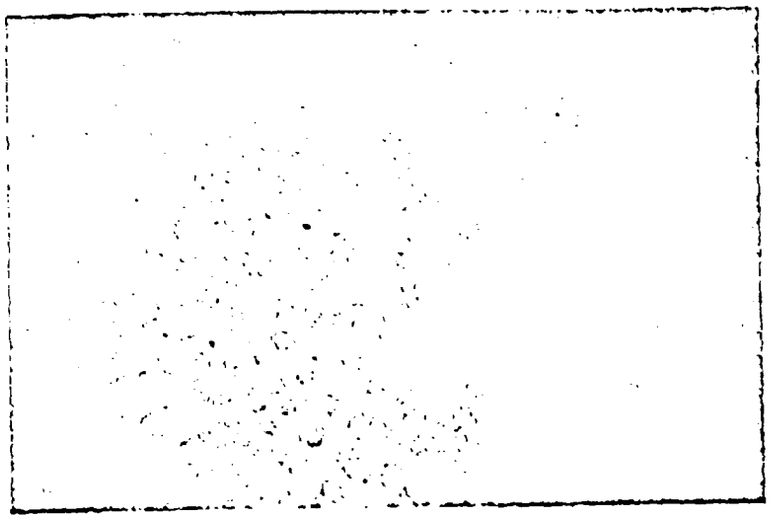

Fig. 3. HeLa cells, 24 hours after infection with vaccine virus. Ob. 40, oc. 7.

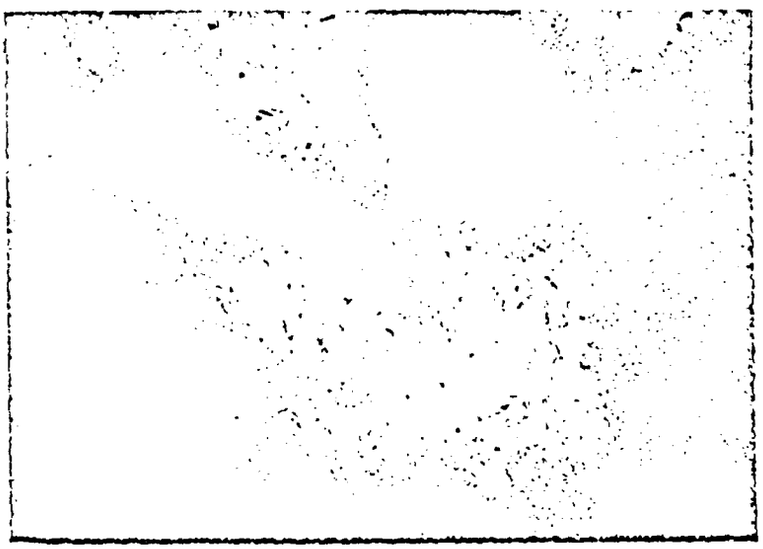

Fig. 4. KB cells, 20 hours after infection with vaccinal virus. Ob. 40, oc. 7.

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